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Association of Ubc9, an E2 ligase for SUMO conjugation, with p53 is regulated by phosphorylation of p53

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Abstract **Small ubiquitin-like modifier-1 (SUMO-1) conjugation to the tumor suppressor protein p53 seems to be regulated by murine double minute 2 homologue (Mdm2). It is thought that the physical association of Mdm2 with p53 is important for the enhancement of SUMO-1 conjugation to p53. However, mutant p53 that does not associate with Mdm2 is still sumoylated, albeit at a reduced level, suggesting that sumoylation of p53 is independent of the presence of Mdm2 and there is a direct association of ubiquitin-conjugating enzyme 9 (Ubc9), an E2 ligase for sumoylation, with p53. Here, we report evidence of the direct interaction of Ubc9 with p53. Furthermore, we observed that the interaction of Ubc9 with p53 was regulated by phosphorylation of p53. In particular, in cells treated with adriamycin that is a DNA damaging agent and that enhances phosphorylation of p53 at Ser-20, SUMO conjugation of p53 was severely impaired possibly by reduced affinity of Ubc9 to p53.**

Keywords: Small ubiquitin-like modifier conjugation; p53; Murine double minute 2 homolog; Ubiquitin-conjugating enzyme 9; Phosphorylation

1. Introduction

The tumor suppressor p53 plays a significant role in the cellular response to genome damage. The importance of p53 in maintaining genome integrity is emphasized by the high frequency of p53 mutations observed in many human tumors [1] and the high rate of tumor development seen in p53 knockout mice [2].

Although the level of p53 in cells is low under normal conditions, exposure to stress signals such as DNA damage and heat shock results in an increase in p53 levels because of its prolonged half-life [3]. The accumulation of p53 is responsible for increased transcription of p53 responsive genes, including proteins involved in cell-cycle regulation (e.g., $p21^{waf1}$) and apoptosis (e.g., Bax) [4–8]. Murine double minute 2 homologue (Mdm2), also a p53 responsive gene, is a critical negative regulator of p53 [9,10].

The ubiquitin-proteasome system is responsible for the degradation and rapid turnover of p53 [11], and this is mediated through the association of p53 with Mdm2 [12–14]. The interaction of Mdm2 and p53 plays a critical role in suppressing the transcriptional activity of p53 [15–17], as well as the ubiquitination and degradation of p53 [18].

The role of ubiquitination in protein degradation is well documented and it has recently become clear that a small ubiquitin-like protein, variously known as small ubiquitin-like modifier 1 (SUMO-1)/sentrin/GMP1/UBL1/PIC1, can be covalently linked to a variety of cellular proteins [19–22]. p53 has been found to be covalently modified by SUMO-1 in vitro and in vivo at lysine 386 [23,24]. This sumoylation is enhanced by the association of E3 ligases, PIAS family proteins [25,26]. However, SUMO-1 and ubiquitin modification do not occur on the same lysine residue in p53 although they do in the case of IkBα [27]. Also, SUMO-1 conjugation to p53 does not seem to alter its transcriptional activity. Some reports suggest increased p53 activity upon sumoylation, but this remains controversial.

Enhanced p53 sumoylation occurs through direct interaction with Mdm2 in cells [28] and a p53 mutant that does not interact with Mdm2 is poorly sumoylated. An Mdm2 mutant with a constitutively activated cryptic nucleolus localization signal targets p53 to the nucleolus and promotes p53 sumoylation. These data suggest that enhanced sumoylation of p53 by Mdm2 is mediated by targeting p53 to the nucleolus through the formation of the Mdm2/p53 complex. Additionally, the phosphorylation state of p53 affects its ability to interact with Mdm2. It is known that DNA-dependent protein kinase, which targets serine residues 15 and 37, reduces the affinity between p53 and Mdm2, and as a consequence, phosphorylated p53 is thought to be a poor substrate for ubiquitination and sumoylation under these conditions.

To clarify the phosphorylation sites on p53 that affects sumoylation, we focused on the Ser-20, a target of the checkpoint kinase 2 (Chk2) activated by DNA damage [29]. In addition, we analyzed the sumoylation of p53 mutants with serine to glutamic acid substitutions at residues 46 or 392, a change thought to mimic the phosphorylated state of p53.

2. Materials and methods

2.1. Cell line

HEK-293T cells (adenovirus-transformed human embryo kidney cell line containing endogenous p53) were maintained in DMEM (Nissui)

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Abbreviations: Mdm2, murine double minute 2 homologue; SUMO, small ubiquitin-like modifier; Ubc9, ubiquitin-conjugating enzyme 9; GST, glutathione *S*-transferase; HA-tag, hemagglutinin epitope tag

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supplemented with 10% fetal calf serum (MBL) and 200 μg/ml of kanamycin (Meiji) at 37 °C in 5% CO_2 atmosphere.

2.2. Plasmid construction

A pcDNA3 (Invitrogen) based plasmid expressing FLAG-tagged human p53 (wild type) was provided by Dr. Y. Ariumi. The p53 mutants were produced individually using the site-directed mutagenesis system, Mutan Super Express Km (Takara), together with the generated pkF18K-p53 as a template. Mutagenetic oligonucleotides (Invitrogen) used in LA-PCR were: p53S20A, 5′-pGGAAACATTTGCAGACCTATG-3′; p53S20E, 5′-pCAGGAAACATTTGAAGACCTATGG-3′; p53S46E, 5′-pGATTTGATGCTG-GAGCCGGACG-3′ and p53S392E, 5′-pCAGAAGGGCCTGACGAAGACTGACATTCTCCAC-3′. All plasmids were sequenced to confirm successful mutagenesis (ABI prism). FLAG-tagged p53 mutants were subcloned into pcDNA3. Plasmids encoding Myc-tagged ubiquitin-conjugating enzyme 9 (Ubc9) and hemagglutinin epitope tag (HA)-tagged SUMO-1 were generated as described previously [30].

2.3. Immunoprecipitations

HEK-293T cells (1×10^5 per 6 cm-diameter dish) were transfected using FuGENE6 (Roche) according to the manufacturer's instructions. To detect the sumoylated forms of p53, cells were lysed in 1 ml of RIPA buffer [25 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM dithiothreitol (DTT), 5 mM ethylenediamine tetra-acetic acid (EDTA), 10 mM *N*-ethylmaleimide, 200 mM indole-3-acetic acid, and a complete protease inhibitor cocktail tablet (Roche)] for 30 min on ice. Cell debris was removed by centrifugation for 15 min. Lysates were pre-cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 h at 4 °C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads-bound proteins were washed four times with RIPA buffer, and immunoprecipitates were eluted and analyzed by immunoblot (IB). For co-immunoprecipitations, cells were transfected with 2 μg pcDNA3-FLAG-p53 expression plasmids with or without 2 μg pcDNA3-Myc-Ubc9 expression plasmid. After 36 h of culture, cells were lysed in 1 ml of immunoprecipitated (IP) buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, and a complete protease inhibitor cocktail tablet]. Lysates were analyzed by immunoprecipitation followed by IB assay.

2.4. Adriamycin treatment

HEK-293T cells were transfected with the expression plasmid encoding FLAG-tagged p53WT. After 24 h of culture, cells were treated with 0.64 μM of adriamycin for another 24 h, and cell lysates were prepared for immunoprecipitation and IB assay.

2.5. Materials

Rat anti-HA (3F10, Roche), mouse anti-Myc (9E10, Santa Cruz), mouse and rabbit anti-FLAG (Sigma) antibodies were purchased. Phospho-Ser20-p53 antibody was generously provided by Dr. Y. Taya. Horseradish peroxidase (HRP)-linked goat antibodies to rat IgG were acquired from Jackson ImmunoResearch Lab. HRP-linked goat antibodies to mouse or rabbit IgG were purchased from Amersham Biosciences. Adriamycin was purchased from Sigma.

3. Results and discussion

To examine the sumoylation of FLAG-tagged p53, plasmids encoding wild-type or mutant p53 and HA-tagged SUMO-1 were transiently co-expressed in cells. Expression of FLAG-tagged p53 was confirmed by immunoprecipitation followed by immunoblotting. Two bands, one with the expected size of wild-type FLAG-p53 and another more slowly migrating band, were detected (Fig. 1). The apparent molecular weight of the upper band was higher in cells exogenously expressing SUMO-1. Because the upper band was detected by anti-HA, this band was the sumoylated form of p53 (Fig. 1, middle panel). Cells expressing p53S46E and p53S392E were similar to wild-type p53, but in the cells producing p53S20E, the upper band was very weak. This was further confirmed by the observation that the upper band in cells expressing p53S20E, but not p53S20A, was detected as a weak signal (Fig. 1, lower panel). These data suggested that phosphorylation of p53 at Ser-20 severely impaired SUMO-1 conjugation. Previously, it was shown that sumoylation of p53 was affected by its interaction with Mdm2 in cells [28]. A p53 mutant that poorly binds Mdm2 undergoes deficient sumoylation. These data suggest that Mdm2 plays an important role in the enhancement of p53 sumoylation. Since a p53 mutant that does not interact with Mdm2 is still sumoylated in vitro, Mdm2 does not seem to be an essential component for the sumoylation of p53 but rather enhances sumoylation. Since Ubc9, the E2 ligase for the SUMO-conjugation reaction, was shown to associate with a target molecule for sumoylation, the association of Ubc9 with

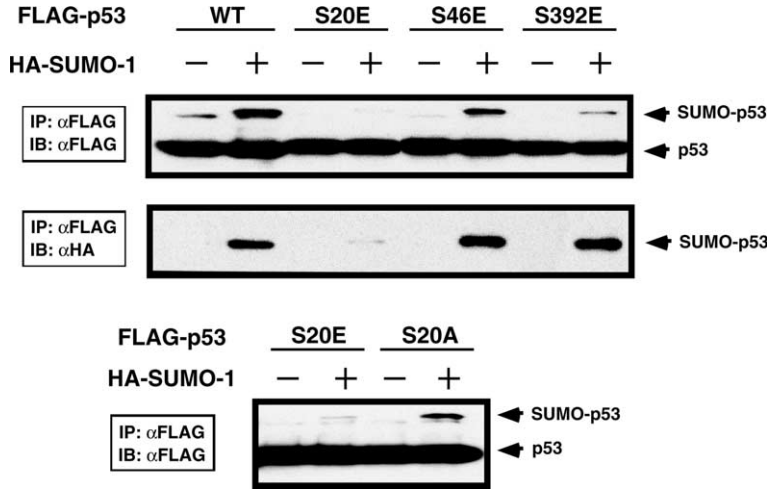

Fig. 1. Phosphorylation mimicked form of p53 at Ser-20 suppresses SUMO-1 conjugation. HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), or the p53 mutants S20E, S20A, S46E, or S392E with (+) or without (−) plasmids expressing HA-SUMO-1. Thirty six hours after transfection, cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates were subjected to 7.5% SDS–PAGE followed by analysis by IB using the anti-FLAG rabbit polyclonal antiserum (top panel). After stripping the filter shown in the top panel, the same filter was re-probed with the anti-HA antibody (middle panel).

p53 was examined. p53 was synthesized by in vitro translation in the presence of ^{35}S-methionine. The interaction of Ubc9 with ^{35}S-labeled wild-type p53, p53S20E and p53S20A was analyzed by GST pull-down analysis (Fig. 2A). Ubc9 bound all the p53 products tested with slightly varying affinity. The affinity of Ubc9 towards p53S20E was reduced by half (Fig. 2B). Because there is no Mdm2 in this assay system, there appears to be a direct interaction between Ubc9. The interaction of Ubc9 and p53 was also observed in vivo (Fig. 2C). Cells were co-transfected with Myc-tagged Ubc9 together with FLAG-tagged p53, p53S20A or p53S20E. Cells lysates prepared from these cells were then analyzed by immunoprecipitation followed by IB. Comparing to the amount of wild-type p53 and p53S20A in the complexes co-precipitated with Ubc9, that of p53S20E was significantly reduced. However, this result may not support the possible direct interaction of p53 with Ubc9, since Ubc9 is known to interact with Mdm2.

The Ser-20 of p53 can be directly phosphorylated by Chk2 in response to DNA damage [29]. To address whether sumoylation of p53 is suppressed by phosphorylation of p53 at Ser-20, we analyzed the sumoylation of p53 after adriamycin treatment. In order to determine whether adriamycin treatment led to phosphorylation of Ser-20 of p53, HEK-293T cells were transfected with a plasmid expressing FLAG-tagged wild-type p53 and treated with 0.64 μM of adriamycin at 24 h post-transfection. Adriamycin treatment was performed for 24 h and the whole cell lysates were prepared for analysis by IB assay with phospho-Ser20 p53 antibody (Fig. 3, upper panel). Ser-20 phosphorylation following adriamycin treatment was observed. We next analyzed sumoylation of p53 in cells treated with adriamycin. Sumoylation of p53 was significantly decreased after adriamycin treatment (Fig. 3, lower panel),

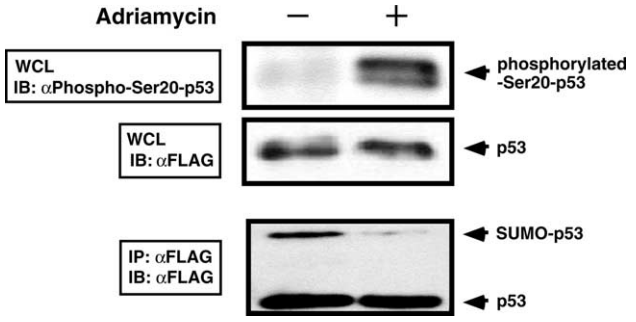

Fig. 3. Sumoylation of p53 is repressed by adriamycin treatment. HEK-293T cells were transfected with 5 μg of plasmid expressing FLAG-tagged wild-type p53. Twenty-four hours after transfection, cells were treated with (+) or without (–) 0.64 μM adriamycin for 24 h, and cell lysates were prepared and IP with anti-FLAG mouse monoclonal antibody. The immunoprecipitates as well as the whole cell lysates were subjected to 7.5% SDS–PAGE and then analyzed by IB using anti-FLAG rabbit polyclonal antiserum.

suggesting that it was repressed by Chk2-mediated phosphorylation of p53 at Ser-20.

It seems that Mdm2 binding to p53 is important for the enhancement of SUMO conjugation to p53 in cells. Since Mdm2 associates with Ubc9, it is possible that Mdm2 enhances the recruitment of Ubc9 to p53. However, this is less likely because sumoylation of p53 in vitro in the presence of Ubc9 was not enhanced by Mdm2. We observed a direct interaction of Ubc9 with p53 in vitro and this interaction was affected by the phosphorylation state of p53. Considering these results and previous reports, it is likely that Ubc9 directly associates and functions to sumoylate p53. Mdm2 may regulate

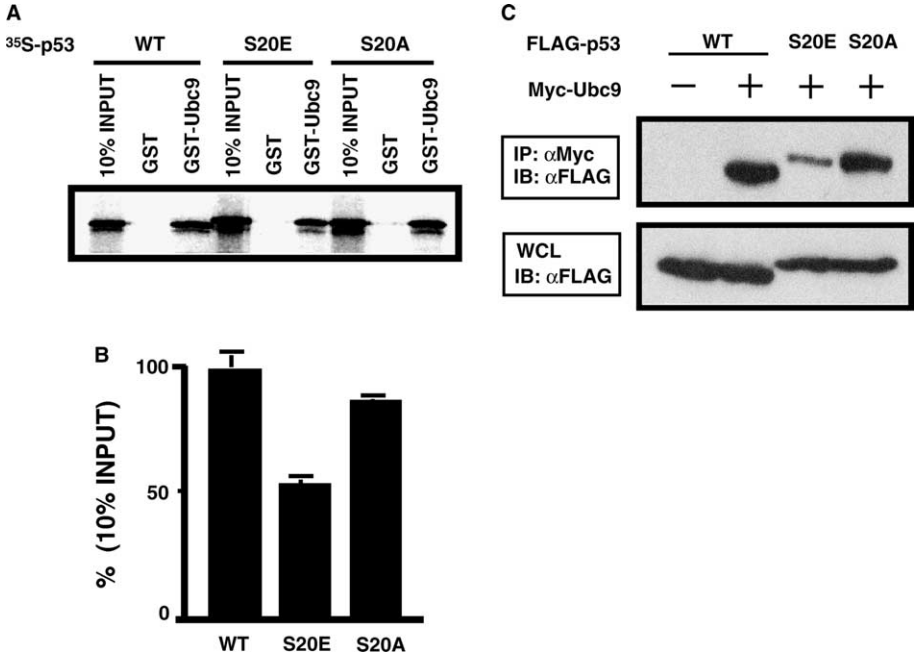

Fig. 2. Association of Ubc9 with wild-type and mutant p53 in vitro and in vivo. (A) ^{35}S-labeled wild-type p53 and p53 mutants, S20E and S20A, were incubated with GST or GST-Ubc9. (B) The GST pull-down complexes were quantitated by imaging analyzer. The experiment was conducted three times independently and data were shown with error bars. (C) HEK-293T cells were transfected with 2 μg of plasmid expressing FLAG-tagged wild-type p53 (WT), S20E, or S20A mutants together with (+) or without (–) Myc-tagged Ubc9 expression plasmid. Thirty six hours after transfection, cell lysates were prepared and subjected to IP with anti-Myc antibody. The immunoprecipitates and the whole cell lysates were subjected to 7.5% SDS–PAGE followed by IB using anti-FLAG rabbit polyclonal antiserum.

this process through at least two mechanisms, enhanced p53 nuclear localization and a mechanism yet to be clarified.

In the present study, we suggest that phosphorylation of p53 at Ser-20 reduces sumoylation. This may result from either the lack of an interaction of p53 with Mdm2 or reduced affinity of Ubc9 to p53 in vivo. Although the physiological roles of sumoylation of p53 are yet to be fully determined, the reduced sumoylation of p53S20E suggests an intrinsic role of sumoylation upon stress induced conditions including DNA damage.

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